Age-induced changes in Mu Opioid Receptor signaling in the midbrain Periaqueductal Gray of male and female rats

Evan F. Fullerton[1], Mary C. Karom[1], and Anne Z. Murphy[1]

[1]Neuroscience Institute, Georgia State University, Atlanta, GA, 30303, USA

Corresponding author:

Anne Z. Murphy, Ph.D.

Neuroscience Institute, Georgia State University

100 Piedmont Ave SE, Atlanta, Georgia, 30303, United States

Tel: 404/413-5332

Email: amurphy@gsu.edu

Declarations of interest: None

Highlights:

- Aged males and females (adult and aged) exhibit reduced vlPAG μ-opioid receptor binding potential compared to adult males
- Aged males and females (adult and aged) exhibit reduced opioid-induced vlPAG G-protein activation compared to adult males
- Aged males and females (adult and aged) exhibit reduced vlPAG MOR mediated cAMP inhibition compared to adult males
- Aged rats (males and females) exhibit increased vlPAG mRNA expression of Regulator of G-Protein Signaling (RGS) proteins RGS4 and RGS9-2 compared to adult rats (males and females)

Abstract: The analgesic effects of opioids are attenuated in aged rats. Opioids such as morphine produce attenuated analgesia in aged rodents compared to adults; however, the neural mechanisms underlying this attenuated response are not yet known. The present study investigated the impact of advanced age and biological sex on opioid signaling in the ventrolateral periaqueductal gray (vlPAG) in the presence of chronic inflammatory pain. Assays measuring mu opioid receptor (MOR) radioligand binding, GTPγS binding, receptor phosphorylation, cAMP inhibition, and regulator of G-protein signaling (RGS) protein expression were performed on vlPAG tissue from adult (2-3mos) and aged (16-18mos), male and female rats. Persistent inflammatory pain was induced by intraplantar injection of Complete Freund's Adjuvant (CFA). Adult males exhibited the highest MOR binding potential and the highest G-protein activation (activation efficiency ratio) in comparison to aged males and females (adult and aged). No impact of advanced age or sex on MOR phosphorylation state was observed. DAMGO-induced cAMP inhibition was highest in the vlPAG of adult males compared to aged males and females (adult and aged). vlPAG levels of RGS4 and RGS9-2, critical for terminating G-protein signaling, were assessed using RNAscope. Adult rats (both males and females) exhibited lower levels of vlPAG RGS4 and RGS9-2 mRNA expression compared to aged males and females. The observed age-related reductions in vlPAG MOR binding potential, G-protein activation efficiency, and cAMP inhibition, along with the observed age-related increases in RGS4 and RGS9-2 vlPAG expression, provide potential mechanisms whereby the response to opioids is attenuated in the aged population. These results have significant implications for pain management in this population.

Keywords: chronic pain; opioid signaling; pharmacology; advanced age; sex differences; mu-opioid receptor

24 **1 Introduction**

25 Chronic pain is a debilitating condition that contributes to mental health disorders, disability, and
26 increased risk of premature death (Domenichiello and Ramsden, 2019; Patel et al., 2013). More than 50%
27 of individuals aged 65 and over suffer from chronic pain, and as the United States population rapidly ages,
28 the development of effective strategies for chronic pain management for this demographic is imperative
29 (Dahlhamer, 2018). Opioids, including morphine and fentanyl, are the most potent and commonly
30 prescribed analgesics for the management of chronic pain (Manchikanti et al., 2010; Sullivan et al., 2010),
31 but effective dosing strategies for the elderly remain ill-defined, due in large part to a dearth of knowledge
32 regarding the impact of advanced age on opioid signaling.

33 Clinical studies examining pain management in the elderly are challenging; co-morbid conditions such as
34 diabetes and high blood pressure, and participant use of concomitant medications affect patient
35 outcomes, contributing to difficulties in the interpretation of results (Naples et al., 2016; Prostran et al.,
36 2016). Additionally, there exist age- and sex-related individual differences in the likelihood of reporting
37 pain in a clinical setting which may lead to misrepresentations of analgesic efficacy (Dampier et al., 2013;
38 Reddy et al., 2012). Particularly, aged populations are known to underreport pain due to fears of
39 institutionalization and concerns with addiction and overdose (Reddy et al., 2012).

40 Using a preclinical model of persistent inflammatory pain (intraplantar Complete Freund's Adjuvant;
41 (CFA)), we have previously reported a significant impact of age and sex on morphine potency. Specifically,
42 we showed that aged rats (18mos) exhibit an attenuated response to morphine compared to adults
43 (2mos), with aged males requiring greater than 2x the concentration of morphine than their adult
44 counterparts (Fullerton et al., 2021). Similar results have been reported in prior preclinical studies,
45 suggesting that aged rodents require higher doses of opioids to produce antinociception (Kavaliers et al.,

46 1983; Kramer and Bodnar, 1986; Webster et al., 1976). The mechanisms underlying the attenuation of
47 opioid efficacy in the aged population are currently unknown.

48 Morphine-induced analgesia is mediated primarily via binding to μ-opioid receptors (MOR), prototypical
49 G-protein coupled receptors (GPCRs) located predominantly on neuronal cell membranes (Martin, 1963;
50 Wolozin and Pasternak, 1981). Following agonist binding, coupled G-proteins undergo a conformational
51 change in which the guanosine diphosphate (GDP) bound to the inactive alpha subunit is replaced by
52 guanosine triphosphate (GTP), catalyzing the subunit and promoting opioid signaling through interaction
53 with downstream effectors (Senese et al., 2020). MORs are coupled to a family of G-proteins called Gi/o
54 that signal via inhibition (Koehl et al., 2018; Bouchet et al., 2021). This G-protein signaling is downregulated
55 or terminated by **R**egulator of **G**-Protein **S**ignaling (RGS) proteins, which act as GTPase activating proteins
56 (GAPS), hydrolyzing the active GTP back into GDP and terminating downstream signaling (Gerber et al.,
57 2016). RGS proteins, particularly RGS4 and RGS9-2, have been previously implicated in opioid-mediated G-
58 protein signaling, and have been shown to attenuate analgesia (Garnier et al., 2003; Psifogeorgou et al.,
59 2007; Avrampou et al., 2019; Senese et al., 2020). A primary target of MOR downstream signaling is the
60 effector adenylate cyclase (AC). AC enzymatically converts adenosine triphosphate (ATP) to the excitatory
61 second messenger cyclic adenosine monophosphate (cAMP). MOR binding inhibits AC, thereby reducing
62 cAMP expression, facilitating neuronal hyperpolarization and promoting analgesia (Santhappan et al.,
63 2015). Like other G-protein coupled receptors, MOR signaling is subject to desensitization via the cellular
64 mechanism of receptor phosphorylation, which limits agonist binding and thus attenuates opioid signaling.
65 (Zhang et al., 1996; Yu et al., 1997; Groer et al., 2011).

66 The present studies test the hypothesis that the observed age-induced reduction in morphine potency in
67 the presence of chronic pain is mediated by changes in MOR signaling within the midbrain periaqueductal
68 gray, a CNS region critical for the opioid modulation of pain. (Basbaum et al., 1976; Behbehani and Fields,

69 1979; Morgan et al., 2006, 1992). The ventrolateral PAG (vlPAG) contains a large population of MOR+
70 neurons, and direct administration of MOR agonists into the PAG produces potent analgesia (Satoh et al.,
71 1983; Jensen and Yaksh, 1986; Bodnar et al., 1988; Loyd et al., 2008) In addition, intra-PAG administration
72 of MOR antagonists or lesions of PAG MOR significantly attenuate the analgesic effect of systemic
73 morphine, suggesting a critical role for PAG MOR in mediating morphine action (Ma and Han, 1991; Y.
74 Zhang et al., 1998; Loyd et al., 2008).

75 We have previously shown that aged rats exhibit reduced vlPAG MOR protein expression and reduced
76 MOR agonist binding in the vlPAG compared to adult rats (Fullerton et al., 2021), suggesting that
77 diminished levels of functioning MOR in the vlPAG contribute to the attenuated opioid potency seen in the
78 aged. The present studies build on these previous findings to assess age and sex differences in MOR
79 availability, affinity and phosphorylation, G-protein activation, cAMP inhibition, and expression of RGS
80 proteins. We report that aged rats, and more specifically males, exhibit reduced MOR binding potential,
81 reduced G-protein activation, reduced cAMP inhibition, and increased RGS regulation compared to adults,
82 with no change in MOR phosphorylation. Similar changes were observed in females regardless of age.
83 These findings identify several mechanisms whereby opioid signaling is attenuated in the aged rat, and
84 provide a framework for the development of novel pharmacological therapies to improve pain
85 management in the elderly.

86 **2 Materials and methods**

87 **2.1 Experimental subjects**

88 Adult (2-3mos) and aged (16-18mos) male and regularly cycling female Sprague–Dawley rats were used
89 in these experiments (Charles River Laboratories, Boston, MA). Rats were co-housed in same-sex pairs
90 on a 12:12 hour light/dark cycle (lights on at 08:00 am). Access to food and water was ad libitum
91 throughout the experiment, except during testing. All studies were approved by the Institutional Animal

92 Care and Use Committee at Georgia State University and performed in compliance with Ethical Issues of
93 the International Association for the Study of Pain and National Institutes of Health. All efforts were
94 made to reduce the number of rats used in these experiments and to minimize pain and suffering. All
95 assays were performed on separate cohorts of rats (n=8/sex/age; N=32), with the exception of the
96 radioligand binding and GTPγS assays, which were run simultaneously on PAG tissue from a single
97 cohort.

98 **2.2 Vaginal cytology**

99 Beginning ten days prior to testing, vaginal lavages were performed daily on adult and aged female rats
100 to confirm that all rats were cycling regularly and to keep daily records of the stage of estrous. Proestrus
101 was identified as a predominance of nucleated epithelial cells, and estrus was identified as a
102 predominance of cornified epithelial cells. Diestrus 1 was differentiated from diestrus 2 by the presence
103 of leukocytes. Rats that appeared between phases were noted as being in the more advanced stage
104 (Loyd et al., 2007).

105 **2.3 CFA-induced chronic pain treatment**

106 72 hours prior to experimentation, persistent inflammatory pain was induced by injection of complete
107 Freund's adjuvant (CFA; Mycobacterium tuberculosis; Sigma; 200µl), suspended in an oil/saline (1:1)
108 emulsion, into the plantar surface of the right hind paw. Edema was present within 24 hours of
109 injection, indicated by a >100% change in paw diameter, determined using calibrated calipers applied
110 midpoint across the plantar surface compared to handled paw.

111 **2.4 Membrane preparation for radioligand binding and GTPγS assays**

112 vlPAG membrane protein lysates were prepared from adult and aged, male and female, handled and
113 CFA treated rats to be used for radioligand binding assay and GTPγS assays. 72 hours post-CFA injection

114 or handling, rats were restrained using DecapiCones and decapitated. Brains were removed rapidly,
115 flash-frozen in 2-methyl butane on dry ice, and stored at −80°C. Ventrolateral PAG tissue from caudal
116 PAG (Bregma -7.5 through -8.5) was dissected from each brain using a straight edge razor at -20°C. On
117 the day of the assay PAG sections were placed in ice cold assay buffer (50 mM Tris-HCl, pH 7.4). Tissue
118 was homogenized with a glass dounce and centrifuged at 20,000g at 4°C for 30 min. The supernatant
119 was discarded and the pellet resuspended in assay buffer (Li et al., 2018; Shaqura et al., 2016b, 2016a;
120 Zollner et al., 2003). Membrane protein concentration was calculated using the Bradford Assay, and
121 lysates of vlPAG membrane protein from adult and aged, male and female, naïve and CFA treated rats
122 (n=4; N=32) were used immediately for radioligand binding assays and GTPγS assays.

123 **2.5 Saturation radioligand binding assay**

124 Saturation binding experiments were performed on vlPAG membranes using [^{3}H]DAMGO (specific
125 activity 50 Ci/mmol, American Radiolabeled Chemicals, Missouri). Briefly, 100 µg membrane proteins
126 were incubated with various concentrations of [^{3}H]DAMGO (0.5 to 5nM), in a total volume of 1ml of
127 binding buffer (50mM Tris-HCl pH 7.4). Nonspecific binding was defined as radioactivity remaining
128 bound in the presence of 10 µM unlabeled naloxone. At the end of the incubation period (1h at RT)
129 bound and free ligands were separated by rapid filtration over Whatman brand Grade GF/C glass filters
130 (Sigma-Aldrich) using a sampling vacuum manifold (MilliporeSigma). Filters were washed four times with
131 5ml of cold dH_20. Bound radioactivity was determined by liquid scintillation spectrophotometry after
132 overnight extraction of the filters in 3ml of scintillation fluid. All experiments were performed in
133 triplicate. B_{max} and K_d values were determined by nonlinear regression analysis of concentration-effect
134 curve in GraphPad Prism 9.1.

135 **2.6 Agonist-stimulated [^{35}S]GTPγS binding**

136 DAMGO-stimulated [^{35}S]GTPγS binding to PAG membrane protein was assessed by incubating
137 membrane protein (100ug) in the presence or absence of [^{35}S]GTPγS (0.1nM) (specific activity 1250
138 Ci/mmol, American Radiolabeled Chemicals, Missouri) and various concentrations of DAMGO (2 to
139 30,000 nM) in assay buffer (50 mM Tris-HCl, pH 7.4) for 30min at 30°C. Stimulated [^{35}S]GTPγS binding
140 was compared to unstimulated binding at each measurement point and presented as percent basal
141 binding. At the end of the incubation period, bound and free ligands were separated by rapid filtration
142 over Whatman brand Grade GF/B glass filters (Sigma-Aldrich) using a sampling vacuum manifold
143 (MilliporeSigma). Filters were washed four times with 5ml of cold buffer (50 mM Tris-HCl, pH 7.4).
144 Bound radioactivity was determined by liquid scintillation spectrophotometry after overnight extraction
145 of the filters in 3ml of scintillation fluid. All experiments were performed in duplicate. Efficacy (Emax) is
146 defined as the maximum percent stimulation by an agonist; potency (EC_{50}) is defined as the
147 concentration of DAMGO required for half the maximal response. Emax and EC_{50} values were
148 determined by nonlinear regression analysis of concentration-effect curves using Graph Pad Prism 9.1.

149 **2.7 Phosphorylated MOR analysis**

150 To assess the impact of advanced age and sex on MOR phosphorylation state, levels of MOR
151 phosphorylation were analyzed by Western blot. The lysis buffers used and general methods are the
152 same as reported in (Lei et al., 2017). Briefly, rat PAG samples were homogenized in ~500 µL of lysis
153 buffer using a glass dounce and rotated overnight at 4°C. The samples were then spun down at 13,000g
154 for 10 min at 4°C. The resulting lysates were quantified by DC Protein Assay Kit (Bio-Rad, #500-0111)
155 according to the manufacturer's instructions. 50-75 µg of soluble protein per sample (same for all
156 samples in a set) was run on 10% Bis-Tris Bolt PAGE gels (Fisher Scientific, #NW00100BOX), and wet-
157 transferred to nitrocellulose membrane (Protran 0.2 µm NC, #45-004-001 from Fisher Scientific) at 30V
158 at 4°C for 2-3 hours. Membranes were blocked using 5% nonfat dry milk in TBS, then blotted for primary

159 antibody target in 5% BSA in TBST overnight rocking at 4°C. Primary antibodies used were: pMOR
160 (1:1000, Bioss #bs-3724R), tMOR (1 µg/mL, R&D #MAB6866), and GAPDH (1:1000, Invitrogen #MA5-
161 15738). All 3 targets were always probed for on the same blot, using low pH stripping buffer between
162 each set. Secondary antibodies were used at 1:5000 each in 5% nonfat dry milk in TBST, rocking for ~1 hr
163 at room temperature. Secondary antibodies used were: Goat-α-Mouse-IRDye800CW (Fisher Scientific
164 #NC9401841) and Goat-α-Mouse-IRDye680LT (Fisher Scientific #NC0046410). Target signal was acquired
165 using an Azure Sapphire imager using the near-infrared channels (658 and 784 nm). Band density was
166 quantified and background subtracted using the onboard AzureSpot analysis software. Samples were
167 run with at least two representatives of each experimental group on the same gel. Adult and aged, male
168 and female, naïve and CFA treated rats (n=4; N=32) were used for these experiments. pMOR signal was
169 normalized to same sample tMOR or GAPDH as indicated, and all samples were normalized to the Adult
170 Male average on each gel before combining data from different gels.
171

172 **2.8 Agonist-dependent cAMP inhibition**

173 DAMGO-dependent cAMP inhibition was analyzed using a cAMP assay. vlPAG membrane protein lysates
174 were prepared from adult and aged, male and female, handled and CFA treated rats. 72 hours post-CFA
175 injection or handling, rats were restrained using DecapiCones and decapitated. Brains were removed
176 rapidly, flash-frozen in 2-methyl butane on dry ice, and stored at −80°C. Ventrolateral PAG tissue from
177 caudal PAG (Bregma -7.5 through -8.5) was dissected from each brain using a straight edge razor at -
178 20°C. Briefly, vlPAG tissues were homogenized in ice-cold lysis buffer containing 0.25-M sucrose, 50-mM
179 Tris–HCl pH 7.5, 5-mM EGTA, 5-mM EDTA, 1-mM phenylmethylsulfonyl fluoride, 0.1-mM dithiothreitol,
180 and 10 µg/ml leupeptin. The homogenized tissues were then centrifuged at 1000×*g* for 5 min (4 °C) and
181 the supernatant was centrifuged at 35000 ×*g* for 10 min (Viganò et al., 2003). vlPAG membrane protein
182 samples (100ug) from adult and aged, male and female, naïve and CFA treated rats (n=4; N=32) were

183 incubated in 1uM forskolin (FSK) and in the presence or absence of 10uM DAMGO to stimulate
184 adenylate cyclase activity. cAMP levels in vlPAG tissue were determined using the LANCE *Ultra* cAMP
185 Detection Kit (Perkin Elmer) a time-resolved fluorescence resonance energy transfer (TR-FRET) cAMP
186 immunoassay. Data is plotted as % change in TR-FRET signal comparing FSK baseline measurements to
187 measurements following DAMGO-stimulated cAMP inhibition.

188 **2.9 Single molecule fluorescence in situ hybridization**

189 Single cell fluorescent in situ hybridization (RNAscope) assays were used to determine mRNA expression
190 of OPRM1, RGS9-2, and RGS4 in the vlPAG. Rats were restrained using DecapiCones and decapitated.
191 Brains were removed rapidly, flash frozen in 2-methyl butane on dry ice, and stored RNase free at −80
192 °C. Frozen tissue was sectioned in a 1:6 series of 20 µm coronal sections at –20 °C with a Leica CM3050S
193 cryostat. Sections were immediately mounted onto Superfrost slides (20 °C) and stored at −80 °C until
194 the time of the assay. vlPAG sections from adult and aged, male and female, naïve and morphine
195 treated rats were used (n=4; N=32. For morphine dosing paradigm see (Fullerton et al., 2021). Tissue
196 was processed for single molecule fluorescence in situ hybridization (smFISH) according to the
197 RNAscope Multiplex Kit protocol (Advanced Cell Diagnostics) using probes for OPRM1, RGS4, and RGS9-
198 2. To facilitate cellular mRNA quantification within the vlPAG, sections were counterstained with DAPI.
199 mRNA puncta were visualized as fluorescent signal. Fluorescent images were captured on Zeiss LSM 700
200 Confocal Microscope at 40x, and mRNA expression (target puncta/DAPI) was calculated using Imaris
201 software. To determine RGS4 and RGS9-2 expression levels in MOR+ neurons, quantification was
202 restricted to puncta located within 10uM of DAPI that co-expressed OPRM1 mRNA. mRNA expression
203 values were determined for the left and right ventrolateral subdivision of each PAG image from two
204 representative levels of the mid-caudal PAG (Bregma −7.74 and −8.00). As there was no significant effect
205 of rostrocaudal level in the analyses, data were collapsed and presented as vlPAG and were averaged for

206 each rat. RGS4 and RGS9-2 mRNA values are expressed as the mean ± standard error of the mean (SEM).
207 All images were collected and analyzed by an experimenter blinded to the experimental condition.

208 **2.10 Statistical analysis and data presentation**

209 All values are reported as mean ± SEM. Data were assessed for normality and homogeneity of variance
210 using Shapiro-Wilk and Bartlett's tests. Significant main effects of sex, age, and treatment were assessed
211 using ANOVA; $p < 0.05$ was considered statistically significant. Tukey's post-hoc tests were conducted to
212 determine significant mean differences between groups that were a priori specified. Data are expressed
213 either as fmol/protein or nm DAMGO for radioligand binding assay, or Emax or median effective
214 concentration for GTPγS assay.

215 **3 Results**

216 **3.1 Advanced age and sex impact vlPAG MOR binding properties**

217 To assess the impact of advanced age, biological sex, and pain vlPAG MOR signaling, we first used
218 radioligand saturation binding assays to determine MOR binding parameters. The saturation binding
219 curves generated with [^{3}H]DAMGO are shown in Fig. 1A. There was no significant impact of treatment
220 on any measures, so CFA and handled groups are combined.

221 Kd values, indicative of receptor affinity, were determined using the concentration-effect curves
222 generated from each sample. Analyses of Kd values indicated no significant impact of age [$F_{(1,26)} = 0.770$,
223 $p=0.388$] or sex [$F_{(1,26)} = 1.241$, $p=0.275$], or a significant interaction [$F_{(1,26)} = 1.278$, $p=0.269$] (Fig. 1B).

224 Analyses of B_{max} values, indicative of receptor availability, showed no significant impact of age [$F_{(1,28)}$ =
225 2.661, $p=0.114$] or sex [$F_{(1,28)} = 0.002$, $p=0.967$], but a significant interaction [$F_{(1,28)} = 4.995$, $p=0.034$]. Post
226 hoc analysis showed a significant difference in vlPAG MOR availability between adult males and aged
227 males, as evidenced by reduced B_{max} values in the aged males compared to their adult counterparts (p =

228 0.025)(Fig. 1C). B_{max} values for aged males were markedly reduced compared to adult males, while adult
229 females and aged females exhibited smaller reductions in B_{max} compared to their adult male
230 counterparts (%Δ -23.6 and -17.1, respectively. Fig. 1C).

231 Analyses of binding potential (BP) values, a measure that takes into consideration the density of
232 available receptors and the affinity of the receptor for its agonist, showed a significant impact of sex,
233 with males exhibiting greater binding potential than females [$F_{(1,28)}$ = 4.631, p=0.040] and a significant
234 interaction between age and sex [$F_{(1,28)}$ = 9.110, p=0.005]. No significant impact of age [$F_{(1,28)}$ = 3.312,
235 p=0.079] was observed. Post hoc analyses showed a significant difference in vlPAG MOR binding
236 potential between adult males and adult females (p = 0.006), and adult males and aged males (p =
237 0.010), indicating that adult males exhibit greater vlPAG MOR binding potential than their aged male
238 counterparts and their adult female counterparts (Fig. 1D). Adult females, aged males, and aged females
239 all exhibited marked reductions in MOR binding potential compared to their adult male counterparts
240 (%Δ -52, -48.7, and -40, respectively; Fig. 1D).

241 **3.2 Advanced age and sex impact opioid-induced G-protein activation in the vlPAG**

242 We next conducted GTPγS binding assays to determine if advanced age or biological sex impacted MOR
243 mediated G-protein activation. Preliminary studies revealed a significant main effect of chronic pain [$F_{(1,}$
244 $_{23)}$ = 21.16, p = 0.0001]. To improve the translatability of these results to the target population of aged
245 patients suffering from chronic pain, GTPγS experiments were performed exclusively on CFA-treated
246 rats. Concentration curves generated from [^{35}S]GTPγS assays are shown in Fig. 2A. Analyses of E_{max}
247 values, a measure of G-protein availability, indicated no significant impact of age [$F_{(1,24)}$ = 0.035, p =
248 0.8532] or sex [$F_{(1,25)}$ = 2.852, p = 0.1042], or a significant interaction [$F_{(1,24)}$ = 2.023, p = 0.1678] (Fig. 2B).

249 Analyses of EC_{50} values, a measure of the concentration of DAMGO required for effective G-protein
250 binding, indicated a significant impact of sex, with males exhibiting lower effective concentration values

251 than females [$F_{(1,24)}$ = 5.177, p = 0.0321]. There was no significant impact of age [$F_{(1,24)}$ = 2.339, p =
252 0.1393], and no significant interaction [$F_{(1,24)}$ = 2.473, p = 0.1289] (Fig. 2C). Compared to their adult male
253 counterparts, aged males, adult females, and aged females all exhibited marked increases in G-protein
254 EC_{50} as evidenced by their percent change values (%Δ 60.5, 114.8, and 98.6, respectively) (Fig. 2C).
255 Together, these results suggest that opioid-induced G-protein signaling is impaired in the PAG of the
256 aged rat and the female rat.

257 Next, we calculated the coefficient ratio of analysis of E_{max} / EC_{50}, [indicative of overall G-protein
258 activation efficiency]. A significant impact of age was noted, with adults exhibiting higher vlPAG G-
259 protein activation efficiency than aged rats [$F_{(1,24)}$ = 6.491, p = 0.0177]. There was also a significant
260 impact of sex, with males exhibiting higher vlPAG G-protein activation efficiency compared to females
261 [$F_{(1,24)}$ =10.39, p = 0.0036]. A significant interaction was also observed [$F_{(1,24)}$ = 13.93, p = 0.0010]. Post
262 hoc analyses showed a significant difference in vlPAG G-protein activation efficiency between adult
263 males and aged males (p = 0.0009), with adult males exhibiting greater vlPAG G-protein activation
264 efficiency than aged males. A significant difference in vlPAG G-protein activation efficiency between
265 adult males and adult females (p= 0.0003) was also observed, with adult males exhibiting greater vlPAG
266 G-protein activation efficiency than adult females (Fig. 2D). Aged males, adult females, and aged females
267 all exhibited marked reductions in G-protein activation efficiency compared to their adult male
268 counterparts (%Δ -40.0, -52.1, and -41.9, respectively; Fig. 2D).

269 **3.3 Phosphorylated MOR**

270 The results above showed significant reductions in MOR binding potential and G-protein activation
271 efficacy. Therefore, we next examined if there was an effect of age and sex on MOR phosphorylation, as
272 increased levels of pMOR would likely contribute to these reductions. Western blots were used to
273 determine the impact of advanced age on MOR phosphorylation at serine-375, a known site of

274 phosphorylation-mediated desensitization (Schulz et al., 2004). There was no significant impact of
275 treatment, so CFA and handled groups are combined. Although increased pMOR/tMOR was observed in
276 aged males compared to their adult counterparts (%Δ 34.0), no significant main effect of age [$F_{(1,41)}$ =
277 2.168, p = 0.1486], sex [$F_{(1,41)}$ = 1.807, p = 0.1863], or age x sex interaction [$F_{(1,41)}$ = 0.4615, p = 0.5008]
278 was observed (Fig. 3).

279

280 **3.4 Advanced age and sex impact opioid-induced cAMP inhibition in the vlPAG**

281 We next determined if advanced age, biological sex, or persistent pain impacted opioid-induced cAMP
282 inhibition in the vlPAG. Forskolin-induced cAMP release was used as a baseline measurement for each
283 group, while forskolin + DAMGO was used to assess the degree to which cAMP was inhibited by
284 DAMGO. Percent change from baseline was used to compare across treatment groups (Fig. 4A). There
285 was no significant impact of persistent pain on any measures, so CFA and handled groups are combined.
286 Our analyses revealed a significant main effect of age [$F_{(1,30)}$ = 9.314, p = 0.0047], a significant main
287 effect of sex [$F_{(1,30)}$ = 10.31, p = 0.0031], and a significant interaction [$F_{(1,30)}$ = 14.24, p = 0.0007]. Post hoc
288 analyses showed a significant difference in cAMP inhibition between adult males and adult females (p =
289 0.0002), adult males and aged males (p = 0.002), and adult males and aged females (p = 0.004),
290 indicating that DAMGO elicits greater cAMP inhibition in adult males compared to aged males and
291 females (both adult and aged) (Fig. 4B).

292 **3.5 Advanced age and sex impact RGS4 and RGS9-2 in the vlPAG**

293 Regulator of G-protein Signaling (RGS) proteins act as GAP accelerators to negatively modulate G-
294 protein signaling. RGS protein family members RGS4 and RGS9-2 are expressed in the vlPAG and have
295 both been shown to regulate opioid signaling by reversing G-protein activation. Therefore, we next used
296 smFISH to determine if RGS4 and RGS9-2 expression in the vlPAG was altered by advanced age and

297 biological sex. In these studies, following CFA administration, a cohort of rats were administered
298 morphine to examine the relationship between morphine EC_{50} and RGS levels. However, no differences
299 were noted between the morphine and naive conditions, so these groups are combined. First, we
300 assessed total vlPAG expression of RGS4 and RGS9-2. These analyses revealed a significant main effect
301 of age on both RGS4 [$F_{(1,37)} = 18.15$, $p = 0.0001$] and RGS9-2 [$F_{(1,41)} = 17.09$, $p < 0.0002$], with aged rats
302 exhibiting increased levels of RGS4 and RGS9-2 mRNA compared to their adult counterparts (Fig. 5A &
303 B). No significant main effect of sex on RGS4 [$F_{(1,37)} = 0.0247$, $p = 0.8763$] or RGS9-2 [$F_{(1,41)} = 0.4855$, $p =$
304 0.4903], or significant interactions between age and sex for RGS4 [$F_{(1,37)} = 0.4994$, $p = 0.4842$] or RGS9-2
305 [$F_{(1,41)} = 0.3246$, $p = 0.5723$] were observed (Fig. 5A & B).

306 Following our assessment of total RGS4 and RGS9-2, we next restricted our analyses to RGS4 and RGS9-2
307 mRNA expressed on MOR+ neurons. Similar to what was noted above, a significant main effect of age on
308 expression of RGS4 [$F_{(1,41)} = 26.47$, $p < 0.0001$] and RGS9-2 [$F_{(1,41)} = 21.69$, $p < 0.0001$] was observed, with
309 aged rats exhibiting increased levels of RGS4 and RGS9-2 mRNA in MOR+ neurons compared to their
310 adult counterparts (Fig. 5C & D). No main effect of sex for RGS4 [$F_{(1,41)} = 0.7881$, $p = 0.3799$] or RGS9-2
311 [$F_{(1,41)} = 0.0006$, $p = 0.9799$], or significant interactions between age and sex for RGS4 [$F_{(1,41)} = 0.6583$, $p =$
312 0.4219] or RGS9-2 [$F_{(1,41)} = 0.0075$, $p = 0.9314$] were observed (Fig. 5C & D).

313

314 **4 Discussion**

315 Aged male rats and female rats, regardless of age, require higher doses of morphine to effectively
316 manage persistent pain compared to their young adult counterparts. The present studies were
317 conducted to determine the impact of advanced age and biological sex on MOR signaling in the vlPAG.
318 Using a clinically relevant model of persistent inflammatory pain, our results show that advanced age
319 causes impairments in several MOR signaling processes in the vlPAG. More specifically, compared to

320 young adult counterparts, aged males show decreased MOR binding potential, G-protein activation, and
321 agonist-stimulated cAMP inhibition, along with increased RGS4 and RGS9-2 expression. Similar results
322 were observed in females, independent of age, and together suggest a model of altered MOR signaling
323 processes in the vlPAG of aged rats (Fig. 6). As MOR binding and downstream signaling are critical for
324 morphine analgesia, the observed age-induced reductions in vlPAG MOR signaling may drive the
325 reduced morphine potency observed in these assays (Fullerton et al., 2021).

326 **Impact of advanced age on MOR binding potential**

327 The present studies are the first to examine the impact of advanced age on MOR signaling in the
328 presence of persistent inflammatory pain in male and female rats. We have previously shown that
329 morphine modulation of persistent inflammatory pain is reduced in aged rats, and that aged animals
330 exhibit reduced DAMGO binding in the vlPAG (Fullerton et al., 2021). In the present study, using
331 saturation radioligand binding assays, we report that adult male rats exhibit greater vlPAG MOR binding
332 potential compared to aged males, and females of either age. This suggests that the decreases in opioid
333 potency seen in the aged males and the females are driven, in part, by reduced activation of vlPAG
334 MOR. Notably, we did not observe any impact of advanced age on the MOR's affinity for its ligand, as
335 there were no significant effects of age or sex on our K_d values. However, aged males had reduced MOR
336 availability as evidenced by lower B_{max} values compared to their adult male counterparts. This result
337 supports our previous finding, that aged males exhibited reduced DAMGO binding, which was assessed
338 using autoradiography on coronal sections through the vlPAG (Fullerton et al., 2021). No impact of
339 persistent inflammatory pain on MOR binding potential was noted, suggesting that persistent
340 inflammatory pain does not alter MOR binding potential in the vlPAG. Previous studies assessing the
341 effects of morphine in the presence and absence of chronic pain support these results, reporting that
342 intra-vlPAG morphine produces thermal antinociception in both handled and CFA exposed rats
343 (Mehalick et al., 2013).

344 The results of the present experiment, taken together with our previous finding of reduced MOR
345 expression using IHC, suggest an age-induced downregulation of the MOR, and a mechanism responsible
346 for the reduced biosynthesis of the receptor in the vlPAG of the aged rat brain. To further assess the
347 age-induced effects on opioid activity in the vlPAG, experimentation assessing MOR signaling controlled
348 for MOR membrane protein by using Bradford Assays and normalizing protein levels across
349 experimental groups. Thus, the analyses of G-protein activation and cAMP inhibition assess for age-
350 induced desensitization of receptor signaling at the molecular level (rather than downregulation) by
351 measuring the impact of advanced age on opioid-induced signaling cascades.

352 These studies used the MOR agonist, DAMGO, which binds MOR with a high affinity. As opioid signaling
353 is known to elicit ligand-dependent regulation (Pan et al., 2005; Narayan et al., 2021), wherein different
354 opioid agonists demonstrate alternative MOR signaling properties (such as G-protein coupling and
355 phosphorylation)(Narayan et al., 2021), our studies cannot account for potential ligand-dependent
356 differences in receptor signaling. Prior research suggests that ligand-dependent regulation can result
357 from the expression of alternative MOR isoforms resulting from alternative splicing of the OPRM1 gene
358 (Pasternak et al., 2004; Oldfield et al., 2008). MOR-1B, a family of MOR isoforms known to be expressed
359 in the brain, are indistinguishable from MOR at the extracellular surface and agonist binding pocket, but
360 elicit alternative signaling properties that affect opioid-mediated cell hyperpolarization. Further,
361 dysregulation of MOR splice variants has been implicated in advanced age (Latorre and Harries, 2017; Li
362 et al., 2017; Wang et al., 2018). Thus, OPRM1 splicing as a function of advanced age may impact
363 downstream opioid signaling, and thus our results from the GTPγS and cAMP experiments.

364 **Impact of advanced age on G-protein activation**

365 Agonist binding at MOR activates Gi/o -proteins and downstream signaling cascades which are
366 necessary for morphine analgesia (Laugwitz et al., 1993; Connor and Christie, 1999; Koehl et al., 2018).

367 We report that adult males exhibit greater G-protein activation efficiency compared to both aged males
368 and adult and aged females. These results suggest that the decreases in opioid potency observed in the
369 aged males and the females are driven, in part, by attenuated G-protein -MOR coupling in the vlPAG. A
370 significant impact of biological sex on G-protein EC_{50} values was observed, with females showing a
371 reduction in the potency of opioid-dependent G-protein activation, as evidenced by increased EC_{50}
372 values. This reduction in vlPAG G-protein activation likely contributes to previous studies reporting that
373 females are less sensitive to the analgesic effects of opioids (Kest et al., 2000; Loyd et al., 2008; Wang et
374 al., 2014; Fullerton et al., 2018). In our initial studies, CFA treated animals exhibited reduced EC_{50} values.
375 Interestingly, this was the only assay in the present study where an impact of CFA treatment was
376 observed. Both clinical and preclinical studies have reported that chronic pain reduces the development
377 of morphine tolerance (Trescot, 2008; Eidson and Murphy, 2013). Our finding that opioid-induced G-
378 protein signaling is amplified in the presence of chronic pain suggests a mechanism whereby CFA
379 treatment attenuates the development of morphine tolerance by bolstering the downstream opioid
380 signaling cascade elicited by G-protein activation and strengthening cellular hyperpolarization.

381 Our finding of attenuated opioid-induced G-protein activation in the aged vlPAG suggests that advanced
382 age promotes changes in opioid signaling at this critical site, but further experimentation is necessary to
383 assess the precise mechanism by which advanced age alters G-protein - MOR signaling. Studies suggest
384 that advanced age induces the downregulation of Gi/o in several brain regions (Young et al., 1991;
385 Alemany et al., 2007; de Oliveira et al., 2019). Age-induced reduction in Gi/o may limit G-protein-MOR
386 coupling and thus contribute to reduced G-protein activation efficiency in the PAG of the aged rat.
387 Studies assessing the impact of advanced age on the expression of Gα isoforms will provide further
388 insight into the effects of age on G-protein-MOR coupling (Lamberts et al., 2011).

389 **Impact of advanced age on MOR Phosphorylation**

390 The results of the radioligand binding and GTPγS assays suggested that aged rats exhibit attenuated
391 MOR-agonist interactions. It has been previously shown that MOR can be desensitized by
392 phosphorylation in its basal state, which limits agonist binding (Zhang et al., 1996; Yu et al., 1997; Groer
393 et al., 2011). Thus, we tested the hypothesis that the reductions in MOR binding potential and G-protein
394 activation efficiency observed in the present study were due to age-induced changes in basal MOR
395 phosphorylation. Our results indicate that while the vlPAG tissue from the aged rats expressed slightly
396 higher levels of MOR phosphorylation at serine-375 compared to the adults, there was no significant
397 impact of advanced age. Thus, the reductions in binding potential observed in the vlPAG of aged rats
398 must be driven by an alternative mechanism.

399 MOR desensitization is mediated by G protein-coupled receptor kinase (GRK)-dependent
400 phosphorylation of the receptor, a mechanism by which β-arrestins bind to MOR, resulting in
401 endocytosis (J. Zhang et al., 1998; Schulz et al., 2004; Dang et al., 2009). Our results suggest no impact of
402 advanced age on GRK-mediated MOR phosphorylation, however, MOR signaling is also desensitized via
403 phosphorylation through the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway (Dang et al.,
404 2009). ERK1/2 phosphorylation stimulates the activity of Gα-interacting protein (GAIP), an RGS protein
405 that acts as a GTPase activator, thereby reducing opioid signaling at the level of G-protein activation
406 (Ogier-Denis et al., 2000). Indeed, pharmacological inhibition of ERK1/2 phosphorylation in a rat model
407 leads to improved morphine analgesia (Popiolek-Barczyk et al., 2014; Melkes et al., 2020). Thus, the age-
408 induced changes in morphine potency may be driven in part by age-induced hyper-phosphorylation of
409 ERK1/2 and resulting downregulation of G-protein signaling.

410 **Impact of advanced age on cAMP inhibition**

411 Agonist binding at MOR elicits a conformational change in the receptor that allows the G-protein α and
412 βγ subunits to interact with downstream effectors. Notably, the Gα subunit binds to adenylate cyclase

413 (AC) and inhibits the conversion of adenosine triphosphate (ATP) to cAMP, thus limiting the activation of
414 cAMP-dependent protein kinase (PKA) and ultimately inducing higher levels of hyperpolarization
415 (Christie, 2008; Santhappan et al., 2015). In the present study, adult males exhibited greater DAMGO-
416 induced cAMP inhibition compared to aged males and adult and aged females, suggesting that aged
417 males and females (adult and aged) elicit attenuated activity of the α subunit at the level of AC, or a
418 weakened relationship between AC and cAMP. This attenuated cAMP inhibition in the aged likely
419 contributes to the observed reduction in opioid potency and may be due, in part, to decreased basal
420 cAMP circulation in the aged brain. Previous studies report age-induced reductions in several regions of
421 the rodent brain including the cortex, thalamus, and hypothalamus (Puri and Volicer, 1981; Titus et al.,
422 2013; Kelly, 2018). In addition to a loss of cAMP circulation, evidence suggests that aged rodents elicit
423 widespread reductions in ATP (Błaszczyk, 2020), which is required for cAMP synthesis.

424 When opioids bind MOR, in parallel to the α subunit interacting with AC and reducing cAMP, the βγ
425 subunits activate G-protein inwardly rectifying potassium (GIRK) channels which hyperpolarize the cell
426 and promote analgesia. The K+ currents elicited by MOR binding are a common method used to
427 measure Gi/o signaling and agonist potency (Lemel et al., 2020; Bouchet et al., 2021). Experimentation
428 that assesses the impact of advanced age on opioid-dependent GIRK channel current, in addition to
429 cAMP, will further reveal the impact of advanced age on this signaling pathway.

430 **Impact of advanced age on the regulation of G-protein signaling by RGS proteins**

431 G-proteins coupled to MOR are activated by MOR agonist binding, resulting in the conversion of GDP
432 into GTP (Senese et al., 2020). This mechanism is essential for G-protein signaling and is modulated by
433 the activity of Regulator of G-protein signaling (RGS) proteins. RGS proteins reduce the magnitude of
434 GPCR signaling via enhancement of GTPase activity on the α subunit of the G-protein complex. By
435 promoting the hydrolysis of the alpha-bound GTP during the active state, GTPases return the α subunit

436 to the GDP-bound inactive state (Roman and Traynor, 2011). In the present study, we observed
437 increased expression of RGS4 and RGS9-2 in the vlPAG of aged animals compared to adults, suggesting
438 greater GTPase activity and thus reduced G-protein signaling in the aged. Similar results were observed
439 when the analysis was limited to MOR+ cells, indicating that opioid-induced G-protein signaling is
440 subjected to greater negative regulation in the vlPAG of the aged rat. These results are consistent with
441 Kim et al. (2005) who reported higher RGS9-2 protein levels in the PAG of 1-year old male rats compared
442 to 3-week-old rats (Kim et al., 2005). Morphine analgesia is increased in mice lacking RGS9-2 (Garzón et
443 al., 2001; Zachariou et al., 2003) and overexpression of RGS4 attenuates MOR signaling (Ippolito et al.,
444 2002). Rivero et al. (2010) reported that advanced age promotes increased expression of RGS4 in the
445 human prefrontal cortex (Rivero et al., 2010). This finding suggests that the age-induced increase in
446 RGS4 in our experiment may not be specific to the vlPAG. Assessment of RGS4 and RGS9-2 expression in
447 multiple brain regions involved in pain processing will further elucidate the mechanism by which
448 advanced age alters opioid-induced G-protein signaling.

449 **Summary**

450 These studies are the first to show that advanced age leads to an attenuation in vlPAG opioid signaling in
451 both male and female rats. Taken together with our previous findings, our results suggest that age-
452 induced reductions in vlPAG MOR expression and binding, combined with attenuated downstream MOR
453 signaling, contribute to the diminished opioid potency seen in aged rats (Fullerton et al., 2021). The
454 results of our analyses demonstrate that aged rats exhibit reduced MOR agonist binding, G-protein
455 activation, and cAMP inhibition, along with increased G-protein regulation by RGS proteins. Thus, these
456 signaling processes are all identified as potential therapeutic targets for improved pain management in
457 the elderly.

458

Funding: This work was supported by the National Institutes of Health [Grant DA041529 AZM, Grant UG3DA047717 JMS]; and Georgia State University [Molecular Basis of Disease Fellowship EFF, Provost's Dissertation Fellowship EFF]

References

Alemany, R., Perona, J.S., Sánchez-Dominguez, J.M., Montero, E., Cañizares, J., Bressani, R., Escribá, P.V., Ruiz-Gutierrez, V., 2007. G protein-coupled receptor systems and their lipid environment in health disorders during aging. Biochim. Biophys. Acta BBA - Biomembr. 1768, 964–975. https://doi.org/10.1016/j.bbamem.2006.09.024

Avrampou, K., Pryce, K.D., Ramakrishnan, A., Sakloth, F., Gaspari, S., Serafini, R.A., Mitsi, V., Polizu, C., Swartz, C., Ligas, B., Richards, A., Shen, L., Carr, F.B., Zachariou, V., 2019. RGS4 Maintains Chronic Pain Symptoms in Rodent Models. J. Neurosci. 39, 8291–8304. https://doi.org/10.1523/JNEUROSCI.3154-18.2019

Basbaum, A.I., Clanton, C.H., Fields, H.L., 1976. Opiate and stimulus-produced analgesia: functional anatomy of a medullospinal pathway. Proc. Natl. Acad. Sci. 73, 4685–4688. https://doi.org/10.1073/pnas.73.12.4685

Behbehani, M.M., Fields, H.L., 1979. Evidence that an excitatory connection between the periaqueductal gray and nucleus raphe magnus mediates stimulation produced analgesia. Brain Res. 170, 85–93. https://doi.org/10.1016/0006-8993(79)90942-9

Błaszczyk, J.W., 2020. Energy Metabolism Decline in the Aging Brain—Pathogenesis of Neurodegenerative Disorders. Metabolites 10, 450. https://doi.org/10.3390/metabo10110450

Bodnar, R.J., Williams, C.L., Lee, S.J., Pasternak, G.W., 1988. Role of mu 1-opiate receptors in supraspinal opiate analgesia: a microinjection study. Brain Res. 447, 25–34. https://doi.org/10.1016/0006-8993(88)90962-6

Bouchet, C.A., McPherson, K.B., Li, M., Traynor, J.R., Ingram, S.L., 2021. Mice Expressing Regulators of G protein Signaling–insensitive Gαo Define Roles of μ Opioid Receptor G α o and G α i Subunit Coupling in Inhibition of Presynaptic GABA Release. Mol. Pharmacol. 100, 217–223. https://doi.org/10.1124/molpharm.121.000249

Christie, M.J., 2008. Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. Br. J. Pharmacol. 154, 384–396. https://doi.org/10.1038/bjp.2008.100

Connor, M., Christie, M.D., 1999. Opioid receptor signalling mechanisms. Clin. Exp. Pharmacol. Physiol. 26, 493–499. https://doi.org/10.1046/j.1440-1681.1999.03049.x

Dahlhamer, J., 2018. Prevalence of Chronic Pain and High-Impact Chronic Pain Among Adults — United States, 2016. MMWR Morb. Mortal. Wkly. Rep. 67. https://doi.org/10.15585/mmwr.mm6736a2

Dampier, C.D., Smith, W.R., Wager, C.G., Kim, H.-Y., Bell, M.C., Miller, S.T., Weiner, D.L., Minniti, C.P., Krishnamurti, L., Ataga, K.I., Eckman, J.R., Hsu, L.L., McClish, D., McKinlay, S.M., Molokie, R., Osunkwo, I., Smith-Whitley, K., Telen, M.J., Sickle Cell Disease Clinical Research Network (SCDCRN), 2013. IMPROVE trial: a randomized controlled trial of patient-controlled analgesia for sickle cell painful episodes: rationale, design challenges, initial experience, and recommendations for future studies. Clin. Trials Lond. Engl. 10, 319–331. https://doi.org/10.1177/1740774513475850

521 Dang, V.C., Napier, I.A., Christie, M.J., 2009. Two Distinct Mechanisms Mediate Acute -Opioid Receptor
522 Desensitization in Native Neurons. J. Neurosci. 29, 3322–3327.
523 https://doi.org/10.1523/JNEUROSCI.4749-08.2009

524 de Oliveira, P.G., Ramos, M.L.S., Amaro, A.J., Dias, R.A., Vieira, S.I., 2019. Gi/o-Protein Coupled Receptors
525 in the Aging Brain. Front. Aging Neurosci. 11. https://doi.org/10.3389/fnagi.2019.00089

526 Domenichiello, A.F., Ramsden, C.E., 2019. The silent epidemic of chronic pain in older adults. Prog.
527 Neuropsychopharmacol. Biol. Psychiatry 93, 284–290. https://doi.org/10.1016/j.pnpbp.2019.04.006

528 Eidson, L.N., Murphy, A.Z., 2013. Persistent peripheral inflammation attenuates morphine-induced
529 periaqueductal gray glial cell activation and analgesic tolerance in the male rat. J. Pain 14, 393–404.
530 https://doi.org/10.1016/j.jpain.2012.12.010

531 Fullerton, E.F., Doyle, H.H., Murphy, A.Z., 2018. Impact of sex on pain and opioid analgesia: a review.
532 Curr. Opin. Behav. Sci. 23, 183–190. https://doi.org/10.1016/j.cobeha.2018.08.001

533 Fullerton, E.F., Rubaharan, M., Karom, M.C., Hanberry, R.I., Murphy, A.Z., 2021. Advanced age
534 attenuates the antihyperalgesic effect of morphine and decreases μ-opioid receptor expression and
535 binding in the rat midbrain periaqueductal gray in male and female rats. Neurobiol. Aging 98, 78–87.
536 https://doi.org/10.1016/j.neurobiolaging.2020.10.020

537 Garnier, M., Zaratin, P.F., Ficalora, G., Valente, M., Fontanella, L., Rhee, M.-H., Blumer, K.J., Scheideler,
538 M.A., 2003. Up-regulation of regulator of G protein signaling 4 expression in a model of neuropathic pain
539 and insensitivity to morphine. J. Pharmacol. Exp. Ther. 304, 1299–1306.
540 https://doi.org/10.1124/jpet.102.043471

541 Garzón, J., Rodríguez-Díaz, M., López-Fando, A., Sánchez-Blázquez, P., 2001. RGS9 proteins facilitate
542 acute tolerance to mu-opioid effects: RGS9 facilitates opioid tachyphylaxis. Eur. J. Neurosci. 13, 801–
543 811. https://doi.org/10.1046/j.0953-816x.2000.01444.x

544 Gerber, K.J., Squires, K.E., Hepler, J.R., 2016. Roles for Regulator of G Protein Signaling Proteins in
545 Synaptic Signaling and Plasticity. Mol. Pharmacol. 89, 273–286. https://doi.org/10.1124/mol.115.102210

546 Groer, C.E., Schmid, C.L., Jaeger, A.M., Bohn, L.M., 2011. Agonist-directed Interactions with Specific β-
547 Arrestins Determine μ-Opioid Receptor Trafficking, Ubiquitination, and Dephosphorylation. J. Biol.
548 Chem. 286, 31731–31741. https://doi.org/10.1074/jbc.M111.248310

549 Ippolito, D.L., Temkin, P.A., Rogalski, S.L., Chavkin, C., 2002. N-terminal Tyrosine Residues within the
550 Potassium Channel Kir3 Modulate GTPase Activity of Gαi. J. Biol. Chem. 277, 32692–32696.
551 https://doi.org/10.1074/jbc.M204407200

552 Jensen, T.S., Yaksh, T.L., 1986. III. Comparison of the antinociceptive action of Mu and delta opioid
553 receptor ligands in the periaqueductal gray matter, medial and paramedial ventral medulla in the rat as
554 studied by the microinjection technique. Brain Res. 372, 301–312. https://doi.org/10.1016/0006-
555 8993(86)91138-8

556 Kavaliers, M., Hirst, M., Teskey, G.C., 1983. Ageing, opioid analgesia and the pineal gland. Life Sci. 32,
557 2279–2287. https://doi.org/10.1016/0024-3205(83)90427-7

558 Kelly, M.P., 2018. Cyclic nucleotide signaling changes associated with normal aging and age-related
559 diseases of the brain. Cell. Signal. 42, 281–291. https://doi.org/10.1016/j.cellsig.2017.11.004

560 Kest, B., Sarton, E., Dahan, A., Fisher, D.M., 2000. Gender Differences in Opioid-mediated Analgesia.
561 Anesthesiology 93, 539–547. https://doi.org/10.1097/00000542-200008000-00034

562 Kim, K.J., Moriyama, K., Han, K.R., Sharma, M., Han, X., Xie, G., Palmer, P.P., 2005. Differential expression
563 of the regulator of G protein signaling RGS9 protein in nociceptive pathways of different age rats. Dev.
564 Brain Res. 160, 28–39. https://doi.org/10.1016/j.devbrainres.2005.08.003

565 Koehl, A., Hu, H., Maeda, S., Zhang, Y., Qu, Q., Paggi, J.M., Latorraca, N.R., Hilger, D., Dawson, R., Matile,
566 H., Schertler, G.F.X., Granier, S., Weis, W.I., Dror, R.O., Manglik, A., Skiniotis, G., Kobilka, B.K., 2018.
567 Structure of the μ-opioid receptor–G i protein complex. Nature 558, 547–552.
568 https://doi.org/10.1038/s41586-018-0219-7

569 Kramer, E., Bodnar, R.J., 1986. Age-related decrements in morphine analgesia: A parametric analysis.
570 Neurobiol. Aging 7, 185–191. https://doi.org/10.1016/0197-4580(86)90041-2

571 Lamberts, J.T., Jutkiewicz, E.M., Mortensen, R.M., Traynor, J.R., 2011. Mu-Opioid Receptor Coupling to
572 Gαo Plays an Important Role in Opioid Antinociception. Neuropsychopharmacology 36, 2041–2053.
573 https://doi.org/10.1038/npp.2011.91

574 Latorre, E., Harries, L.W., 2017. Splicing regulatory factors, ageing and age-related disease. Ageing Res.
575 Rev. 36, 165–170. https://doi.org/10.1016/j.arr.2017.04.004

576 Laugwitz, K.L., Offermanns, S., Spicher, K., Schultz, G., 1993. mu and delta opioid receptors differentially
577 couple to G protein subtypes in membranes of human neuroblastoma SH-SY5Y cells. Neuron 10, 233–
578 242. https://doi.org/10.1016/0896-6273(93)90314-h

579 Lei, W., Mullen, N., McCarthy, S., Brann, C., Richard, P., Cormier, J., Edwards, K., Bilsky, E.J., Streicher,
580 J.M., 2017. Heat-shock protein 90 (Hsp90) promotes opioid-induced anti-nociception by an ERK
581 mitogen-activated protein kinase (MAPK) mechanism in mouse brain. J. Biol. Chem. 292, 10414–10428.
582 https://doi.org/10.1074/jbc.M116.769489

583 Lemel, L., Lane, J.R., Canals, M., 2020. GRKs as Key Modulators of Opioid Receptor Function. Cells 9,
584 2400. https://doi.org/10.3390/cells9112400

585 Li, H., Wang, Z., Ma, T., Wei, G., Ni, T., 2017. Alternative splicing in aging and age-related diseases.
586 Transl. Med. Aging 1, 32–40. https://doi.org/10.1016/j.tma.2017.09.005

587 Li, X., Shaqura, M., Mohamed, D., Beyer, A., Yamada, S., Mousa, S.A., Schäfer, M., 2018. Pro- versus
588 Antinociceptive Nongenomic Effects of Neuronal Mineralocorticoid versus Glucocorticoid Receptors
589 during Rat Hind Paw Inflammation. Anesthesiology 128, 796–809.
590 https://doi.org/10.1097/ALN.0000000000002087

591 Loyd, D.R., Morgan, M.M., Murphy, A.Z., 2007. Morphine Preferentially Activates the Periaqueductal
592 Gray –Rostral Ventromedial Medullary Pathway in the Male Rat: A Potential Mechanism for Sex
593 Differences in Antinociception. Neuroscience 147, 456–468.
594 https://doi.org/10.1016/j.neuroscience.2007.03.053

595 Loyd, D.R., Wang, X., Murphy, A.Z., 2008. Sex Differences in μ-Opioid Receptor Expression in the Rat
596 Midbrain Periaqueductal Gray Are Essential for Eliciting Sex Differences in Morphine Analgesia. J.
597 Neurosci. 28, 14007–14017. https://doi.org/10.1523/JNEUROSCI.4123-08.2008

598 Ma, Q.P., Han, J.S., 1991. Naloxone blocks the release of opioid peptides in periaqueductal gray and N.
599 accumbens induced by intra-amygdaloid injection of morphine. Peptides 12, 1235–1238.
600 https://doi.org/10.1016/0196-9781(91)90200-9

601 Manchikanti, L., Fellows, B., Ailinani, H., Pampati, V., 2010. Therapeutic use, abuse, and nonmedical use
602 of opioids: a ten-year perspective. Pain Physician 13, 401–435.

603 Martin, W.R., 1963. Analgesic and antipyretic drugs in Physiological Pharmacology. Pharmacol Rev,
604 Academic Press, NY 275–312.

605 Mehalick, M.L., Ingram, S.L., Aicher, S.A., Morgan, M.M., 2013. Chronic inflammatory pain prevents
606 tolerance to the antinociceptive effect of morphine microinjected into the ventrolateral periaqueductal
607 gray of the rat. J. Pain 14, 1601–1610. https://doi.org/10.1016/j.jpain.2013.08.003

608 Melkes, B., Markova, V., Hejnova, L., Novotny, J., 2020. β-Arrestin 2 and ERK1/2 Are Important
609 Mediators Engaged in Close Cooperation between TRPV1 and μ-Opioid Receptors in the Plasma
610 Membrane. Int. J. Mol. Sci. 21, 4626. https://doi.org/10.3390/ijms21134626

611 Morgan, M.M., Fossum, E.N., Stalding, B.M., King, M.M., 2006. Morphine antinociceptive potency on
612 chemical, mechanical, and thermal nociceptive tests in the rat. J. Pain 7, 358–366.
613 https://doi.org/10.1016/j.jpain.2005.12.009

614 Morgan, M.M., Heinricher, M.M., Fields, H.L., 1992. Circuitry linking opioid-sensitive nociceptive
615 modulatory systems in periaqueductal gray and spinal cord with rostral ventromedial medulla.
616 Neuroscience 47, 863–871. https://doi.org/10.1016/0306-4522(92)90036-2

617 Naples, J.G., Gellad, W.F., Hanlon, J.T., 2016. The Role of Opioid Analgesics in Geriatric Pain
618 Management. Clin. Geriatr. Med. 32, 725–735. https://doi.org/10.1016/j.cger.2016.06.006

619 Narayan, A., Hunkele, A., Xu, J., Bassoni, D.L., Pasternak, G.W., Pan, Y.-X., 2021. Mu Opioids Induce
620 Biased Signaling at the Full-Length Seven Transmembrane C-Terminal Splice Variants of the mu Opioid
621 Receptor Gene, Oprm1. Cell. Mol. Neurobiol. 41, 1059–1074. https://doi.org/10.1007/s10571-020-
622 00973-5

623 Ogier-Denis, E., Pattingre, S., El Benna, J., Codogno, P., 2000. Erk1/2-dependent phosphorylation of
624 Galpha-interacting protein stimulates its GTPase accelerating activity and autophagy in human colon
625 cancer cells. J. Biol. Chem. 275, 39090–39095. https://doi.org/10.1074/jbc.M006198200

626 Oldfield, S., Braksator, E., Rodriguez-Martin, I., Bailey, C.P., Donaldson, L.F., Henderson, G., Kelly, E.,
627 2008. C-terminal splice variants of the mu-opioid receptor: existence, distribution and functional
628 characteristics. J. Neurochem. 104, 937–945. https://doi.org/10.1111/j.1471-4159.2007.05057.x

629 Pan, Y.-X., Xu, J., Bolan, E., Moskowitz, H.S., Xu, M., Pasternak, G.W., 2005. Identification of four novel
630 exon 5 splice variants of the mouse mu-opioid receptor gene: functional consequences of C-terminal
631 splicing. Mol. Pharmacol. 68, 866–875. https://doi.org/10.1124/mol.105.011858

632 Pasternak, D.A., Pan, L., Xu, J., Yu, R., Xu, M.-M., Pasternak, G.W., Pan, Y.-X., 2004. Identification of three
633 new alternatively spliced variants of the rat mu opioid receptor gene: dissociation of affinity and
634 efficacy. J. Neurochem. 91, 881–890. https://doi.org/10.1111/j.1471-4159.2004.02767.x

635 Patel, K.V., Guralnik, J.M., Dansie, E.J., Turk, D.C., 2013. Prevalence and Impact of Pain among Older
636 Adults in the United States: Findings from the 2011 National Health and Aging Trends Study. Pain 154.
637 https://doi.org/10.1016/j.pain.2013.07.029

638 Popiolek-Barczyk, K., Makuch, W., Rojewska, E., Pilat, D., Mika, J., 2014. Inhibition of intracellular
639 signaling pathways NF-κB and MEK1/2 attenuates neuropathic pain development and enhances
640 morphine analgesia. Pharmacol. Rep. PR 66, 845–851. https://doi.org/10.1016/j.pharep.2014.05.001

641 Prostran, M., Vujović, K.S., Vučković, S., Medić, B., Srebro, D., Divac, N., Stojanović, R., Vujović, A.,
642 Jovanović, L., Jotić, A., Cerovac, N., 2016. Pharmacotherapy of Pain in the Older Population: The Place of
643 Opioids. Front. Aging Neurosci. 8. https://doi.org/10.3389/fnagi.2016.00144

644 Psifogeorgou, K., Papakosta, P., Russo, S.J., Neve, R.L., Kardassis, D., Gold, S.J., Zachariou, V., 2007. RGS9-
645 2 is a negative modulator of μ-opioid receptor function: RGS9-2 modulates μ-opioid receptor
646 responsiveness. J. Neurochem. 103, 617–625. https://doi.org/10.1111/j.1471-4159.2007.04812.x

647 Puri, S.K., Volicer, L., 1981. Age-related changes of cyclic nucleotide levels in rat brain regions. Mech.
648 Ageing Dev. 15, 239–242. https://doi.org/10.1016/0047-6374(81)90132-9

649 Reddy, K.S.K., Naidu, M.U.R., Rani, P.U., Rao, T.R.K., 2012. Human experimental pain models: A review of
650 standardized methods in drug development. J. Res. Med. Sci. Off. J. Isfahan Univ. Med. Sci. 17, 587–595.

651 Rivero, G., Gabilondo, A.M., García-Sevilla, J.A., La Harpe, R., Morentín, B., Javier Meana, J., 2010.
652 Characterization of regulators of G-protein signaling RGS4 and RGS10 proteins in the postmortem
653 human brain. Neurochem. Int. 57, 722–729. https://doi.org/10.1016/j.neuint.2010.08.008

654 Roman, D.L., Traynor, J.R., 2011. Regulators of G Protein Signaling (RGS) Proteins as Drug Targets:
655 Modulating G-Protein-Coupled Receptor (GPCR) Signal Transduction: Miniperspective. J. Med. Chem. 54,
656 7433–7440. https://doi.org/10.1021/jm101572n

657 Santhappan, R., Crowder, A.T., Gouty, S., Cox, B.M., Côté, T.E., 2015. Mu opioid receptor activation
658 enhances regulator of G protein signaling 4 association with the mu opioid receptor/G protein complex
659 in a GTP-dependent manner. J. Neurochem. 135, 76–87. https://doi.org/10.1111/jnc.13222

660 Satoh, M., Kubota, A., Iwama, T., Wada, T., Yasui, M., Fujibayashi, K., Takagi, H., 1983. Comparison of
661 analgesic potencies of mu, delta and kappa agonists locally applied to various CNS regions relevant to
662 analgesia in rats. Life Sci. 33 Suppl 1, 689–692. https://doi.org/10.1016/0024-3205(83)90596-9

663 Schulz, S., Mayer, D., Pfeiffer, M., Stumm, R., Koch, T., Höllt, V., 2004. Morphine induces terminal μ-
664 opioid receptor desensitization by sustained phosphorylation of serine-375. EMBO J. 23, 3282–3289.
665 https://doi.org/10.1038/sj.emboj.7600334

666 Senese, N.B., Kandasamy, R., Kochan, K.E., Traynor, J.R., 2020. Regulator of G-Protein Signaling (RGS)
667 Protein Modulation of Opioid Receptor Signaling as a Potential Target for Pain Management. Front. Mol.
668 Neurosci. 13. https://doi.org/10.3389/fnmol.2020.00005

669 Shaqura, M., Li, X., Al-Khrasani, M., Shakibaei, M., Tafelski, S., Fürst, S., Beyer, A., Kawata, M., Schäfer,
670 M., Mousa, S.A., 2016a. Membrane-bound glucocorticoid receptors on distinct nociceptive neurons as
671 potential targets for pain control through rapid non-genomic effects. Neuropharmacology 111, 1–13.
672 https://doi.org/10.1016/j.neuropharm.2016.08.019

673 Shaqura, M., Li, X., Al-Madol, M.A., Tafelski, S., Beyer-Koczorek, A., Mousa, S.A., Schäfer, M., 2016b.
674 Acute mechanical sensitization of peripheral nociceptors by aldosterone through non-genomic
675 activation of membrane bound mineralocorticoid receptors in naive rats. Neuropharmacology 107, 251–
676 261. https://doi.org/10.1016/j.neuropharm.2016.03.032

677 Sullivan, M.D., Edlund, M.J., Fan, M.-Y., DeVries, A., Braden, J.B., Martin, B.C., 2010. Risks for possible
678 and probable opioid misuse among recipients of chronic opioid therapy in commercial and medicaid
679 insurance plans: The TROUP Study. Pain 150, 332–339. https://doi.org/10.1016/j.pain.2010.05.020

680 Titus, D.J., Furones, C., Kang, Y., Atkins, C.M., 2013. Age-dependent alterations in cAMP signaling
681 contribute to synaptic plasticity deficits following traumatic brain injury. Neuroscience 231, 182–194.
682 https://doi.org/10.1016/j.neuroscience.2012.12.002

683 Trescot, A.M., 2008. Effectiveness of Opioids in the Treatment ofChronic Non-Cancer Pain. Pain
684 Physician 2s;11, S181–S200. https://doi.org/10.36076/ppj.2008/11/S181

685 Viganò, D., Rubino, T., Di Chiara, G., Ascari, I., Massi, P., Parolaro, D., 2003. μ opioid receptor signaling in
686 morphine sensitization. Neuroscience 117, 921–929. https://doi.org/10.1016/S0306-4522(02)00825-4

687 Wang, K., Wu, D., Zhang, H., Das, A., Basu, M., Malin, J., Cao, K., Hannenhalli, S., 2018. Comprehensive
688 map of age-associated splicing changes across human tissues and their contributions to age-associated
689 diseases. Sci. Rep. 8, 10929. https://doi.org/10.1038/s41598-018-29086-2

690 Wang, Y.-J., Huang, P., Blendy, J.A., Liu-Chen, L.-Y., 2014. Brain region- and sex-specific alterations in
691 DAMGO-stimulated [^{35} S]GTPγS binding in mice with *Oprm1* A112G: A118G SNP on MOPR activation.
692 Addict. Biol. 19, 354–361. https://doi.org/10.1111/j.1369-1600.2012.00484.x

693 Webster, G.W., Shuster, L., Eleftheriou, B.E., 1976. Morphine analgesia in mice of different ages. Exp.
694 Aging Res. 2, 221–233. https://doi.org/10.1080/03610737608257178

695 Wolozin, B.L., Pasternak, G.W., 1981. Classification of multiple morphine and enkephalin binding sites in
696 the central nervous system. Proc. Natl. Acad. Sci. 78, 6181–6185.
697 https://doi.org/10.1073/pnas.78.10.6181

698 Young, L.T., Warsh, J.J., Li, P.P., Siu, K.P., Becker, L., Gilbert, J., Hornykiewicz, O., Kish, S.J., 1991.
699 Maturational and aging effects on guanine nucleotide binding protein immunoreactivity in human brain.
700 Brain Res. Dev. Brain Res. 61, 243–248. https://doi.org/10.1016/0165-3806(91)90137-8

701 Yu, Y., Zhang, L., Yin, X., Sun, H., Uhl, G.R., Wang, J.B., 1997. μ Opioid Receptor Phosphorylation,
702 Desensitization, and Ligand Efficacy*. J. Biol. Chem. 272, 28869–28874.
703 https://doi.org/10.1074/jbc.272.46.28869

704 Zachariou, V., Georgescu, D., Sanchez, N., Rahman, Z., DiLeone, R., Berton, O., Neve, R.L., Sim-Selley, L.J.,
705 Selley, D.E., Gold, S.J., Nestler, E.J., 2003. Essential role for RGS9 in opiate action. Proc. Natl. Acad. Sci.
706 100, 13656–13661. https://doi.org/10.1073/pnas.2232594100

707 Zhang, J., Ferguson, S.S.G., Barak, L.S., Bodduluri, S.R., Laporte, S.A., Law, P.-Y., Caron, M.G., 1998. Role
708 for G protein-coupled receptor kinase in agonist-specific regulation of -opioid receptor responsiveness.
709 Proc. Natl. Acad. Sci. 95, 7157–7162. https://doi.org/10.1073/pnas.95.12.7157

710 Zhang, L., Yu, Y., Mackin, S., Weight, F.F., Uhl, G.R., Wang, J.B., 1996. Differential μ Opiate Receptor
711 Phosphorylation and Desensitization Induced by Agonists and Phorbol Esters (∗). J. Biol. Chem. 271,
712 11449–11454. https://doi.org/10.1074/jbc.271.19.11449

713 Zhang, Y., Du, L.N., Wu, G.C., Cao, X.D., 1998. Modulation of intrathecal morphine-induced
714 immunosuppression by microinjection of naloxone into periaqueductal gray. Zhongguo Yao Li Xue Bao
715 19, 519–522.

716 Zollner, C., Shaqura, M.A., Bopaiah, C.P., Mousa, S., Stein, C., Schafer, M., 2003. Painful inflammation-
717 induced increase in mu-opioid receptor binding and G-protein coupling in primary afferent neurons.
718 Mol. Pharmacol. 64, 202–210. https://doi.org/10.1124/mol.64.2.202

719

720

721

722

723

724

725

726

727

728

729

730 **Figure Legends**

731 **Fig. 1.** Saturation binding curve of bound [3H]DAMGO (A). No significant effect of age or sex was
732 observed in MOR affinity (K_d) (B). MOR availability (B_{max}) was significantly lower in aged males compared
733 to adult males (C). Adult females, aged males, and aged females exhibited reduced B_{max} values
734 compared to adult males, indicating reduced MOR availability (C). Adult females, aged males, and aged
735 females exhibited reduced MOR binding potential (B_{max}/K_d) compared to adult males, indicating
736 attenuated agonist interaction at the receptor level (D). ns, not significant. *Significant difference
737 between adult and aged males, or adult males and adult females; $p<0.05$ calculated by Tukey's post hoc
738 test. Graphs indicate mean ± SEM. Values indicate % change from adult male.

739 **Fig 2.** [^{35}S]GTPγS binding curve of agonist-stimulated GTPγS binding (A). No significant effect of age or
740 sex was observed in E_{max} (B). Aged males, adult females, and aged females exhibited increased EC_{50}
741 values compared to adult males, indicating that adult males have the greatest potency (C). Aged males,
742 adult females, and aged females exhibited reduced G-protein activation efficiency (E_{max}/EC_{50}) compared
743 to adult males, indicating attenuated G-protein signaling (D). ns, not significant. *Significant difference
744 between adult and aged males, or adult males and adult females; $p<0.05$ calculated by Tukey's post hoc
745 test. Graphs indicate mean ± SEM. Values indicate % change from adult male.

746 **Fig 3.** No significant impact of advanced age or biological sex on the ratio of phosphorylated MOR/
747 tagged MOR. ns, not significant. Graph indicates mean ± SEM. Values indicate % change from adult
748 male.

749 **Fig 4.** TR-FRET immunoassay showing % change from FSK stimulated baseline and DAMGO inhibited
750 cAMP (circulating cAMP is inversely proportional to TR-FRET signal). Adult males exhibited the highest
751 level of agonist-dependent % change in cAMP (A). Aged males and females exhibited reduced agonist-
752 dependent cAMP inhibition compared to adult males, indicating attenuated downstream opioid
753 signaling (B). *Significant difference between adult and aged males, or adult males and adult females;
754 $p<0.05$ calculated by Tukey's post hoc test. Graphs indicate mean ± SEM. Values indicate % change from
755 FSK baseline.

756 **Fig 5.** mRNA expression of RGS4 (A) and RGS9-2 (B) in the vlPAG was significantly greater in aged rats
757 compared to adults. mRNA expression of RGS4 (C) and RGS9-2 (D) on MOR+ cells in the vlPAG was
758 significantly greater in aged rats compared to adults. (E) Photomicrograph of RGS expression compared
759 an adult male and an aged male PAG section. *Significant difference between adults and aged rats;
760 $p<0.05$ calculated by 2x2 ANOVA. Graphs indicate mean ± SEM.

761 **Fig 6.** Model and table of MOR signaling impairments in the vlPAG of the aged rat. Aged rats exhibit
762 decreased MOR binding potential, decreased G-protein activation, and decreased cAMP inhibition, along
763 with increased RGS expression. No impact of age was found in phosphorylation of MOR at serine-375

764

Figure 1

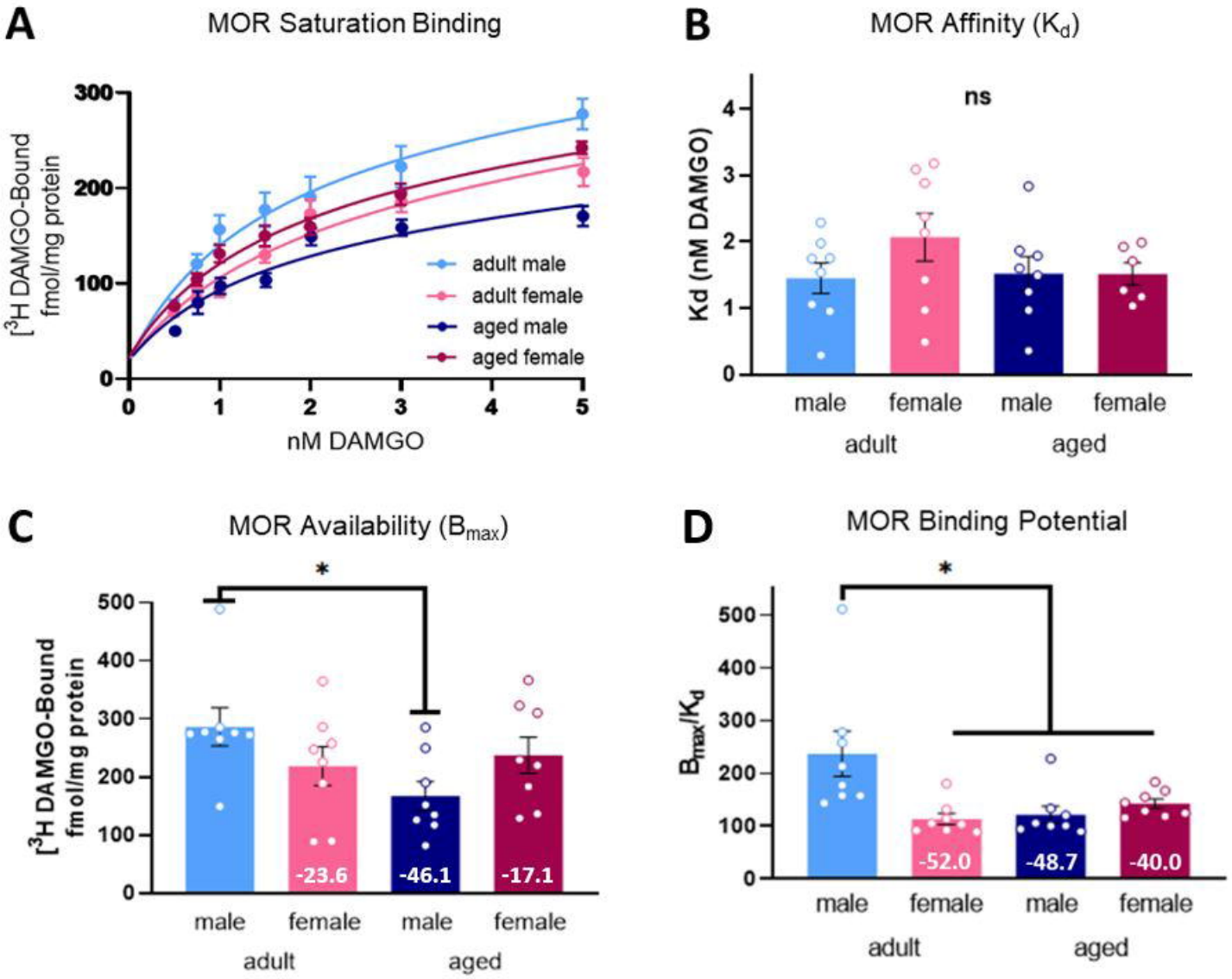

A
MOR Saturation Binding
[3H DAMGO-Bound fmol/mg protein
300
200
100
0
0
1
2
3
4
5
nM DAMGO
adult male
adult female
aged male
aged female
B
MOR Affinity (Kd)
Kd (nM DAMGO)
ns
4
3
2
1
0
male
female
male
female
adult
aged
C
MOR Availability (Bmax)
[3H DAMGO-Bound fmol/mg protein
*
500
400
300
200
100
0
-23.6
-46.1
-17.1
male
female
male
female
adult
aged
D
MOR Binding Potential
Bmax/Kd
*
500
400
300
200
100
0
-52.0
-48.7
-40.0
male
female
male
female
adult
aged

Figure 2

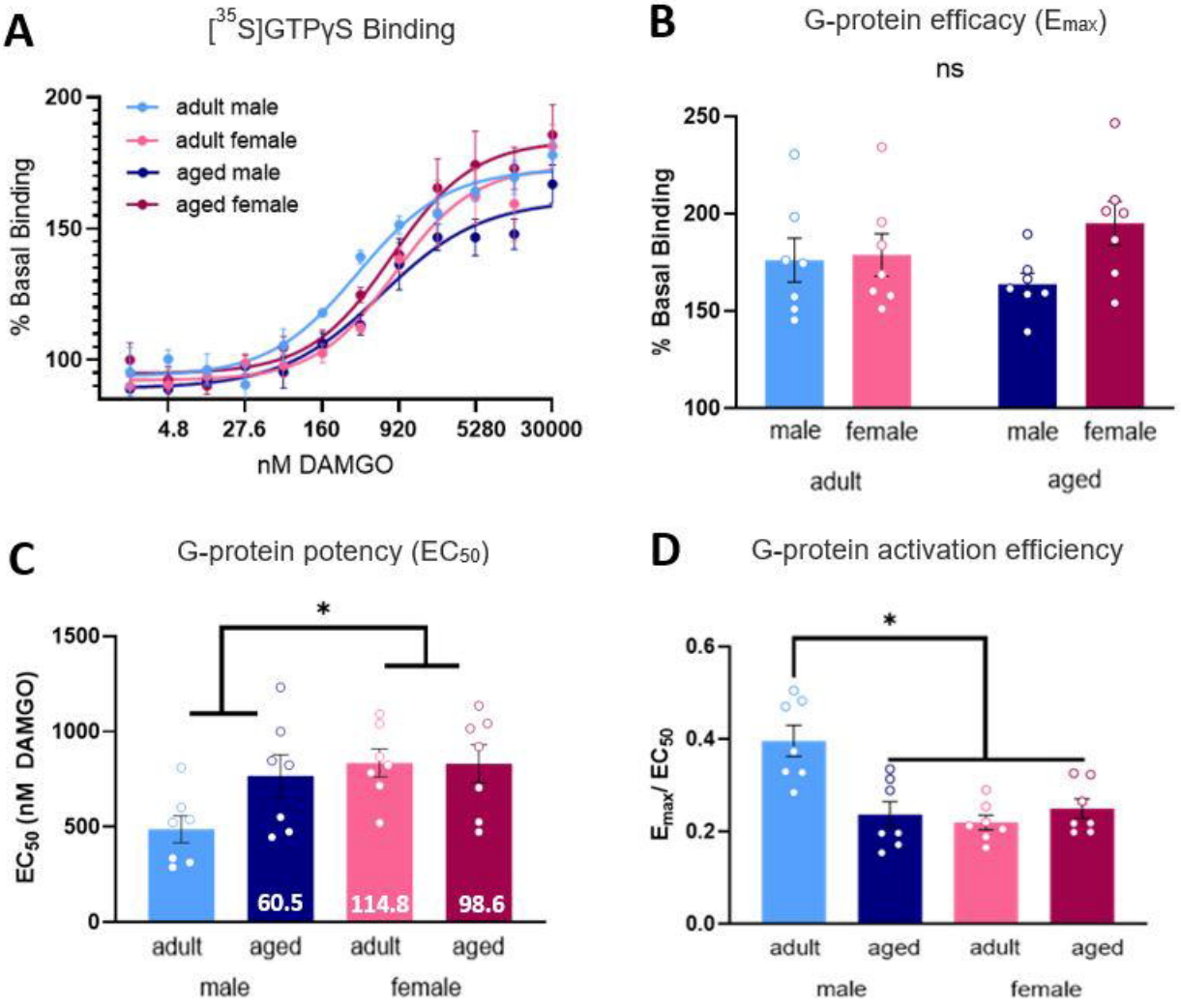

A
[35S]GTPγS Binding
adult male
adult female
aged male
aged female
% Basal Binding
200
150
100
4.8
27.6
160
920
5280
30000
nM DAMGO
B
G-protein efficacy (Emax)
ns
% Basal Binding
250
200
150
100
male
female
male
female
adult
aged
C
G-protein potency (EC50)
*
EC50 (nM DAMGO)
1500
1000
500
0
60.5
114.8
98.6
adult
aged
adult
aged
male
female
D
G-protein activation efficiency
*
Emax/ EC50
0.6
0.4
0.2
0.0
adult
aged
adult
aged
male
female

Figure 3

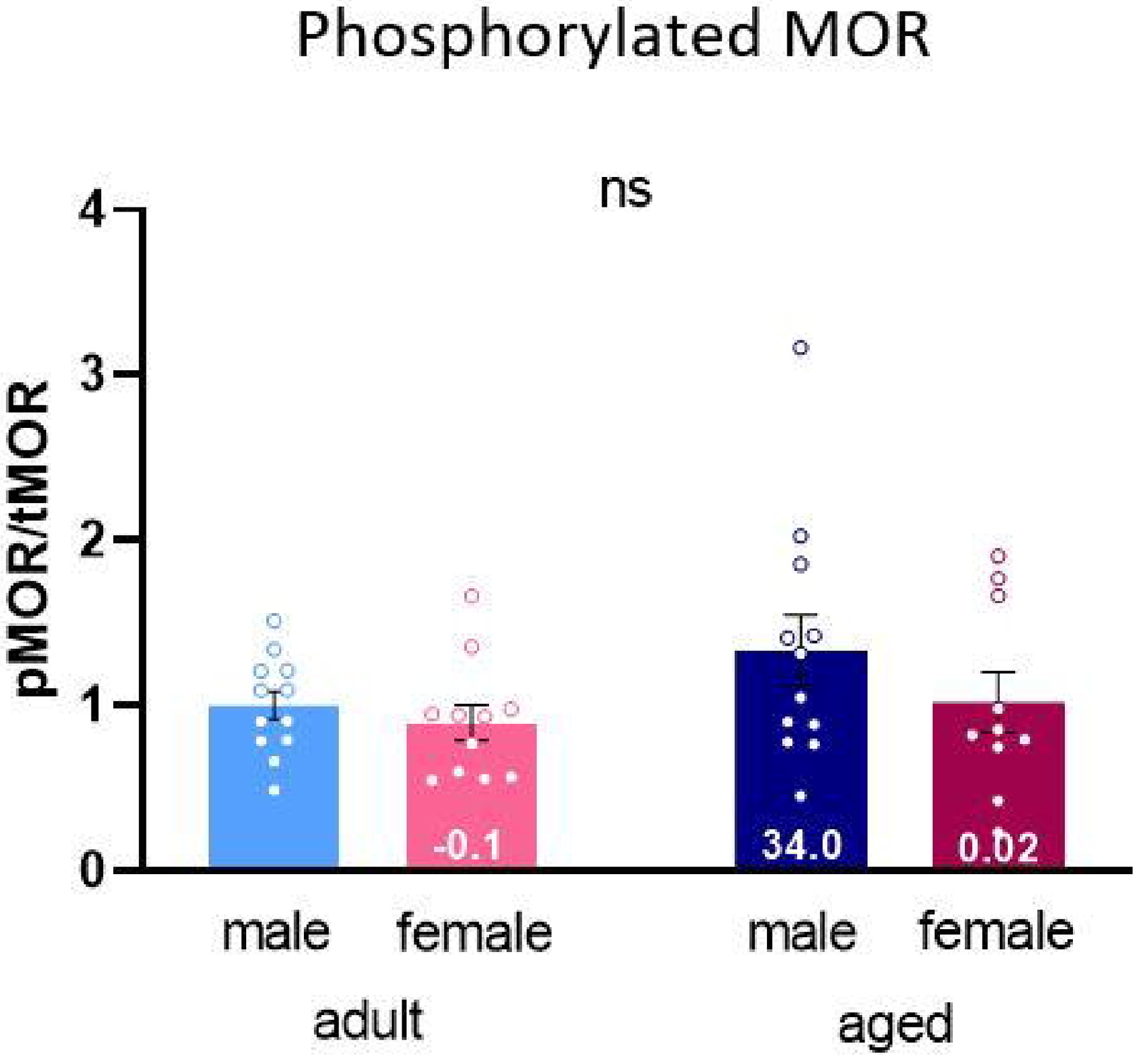
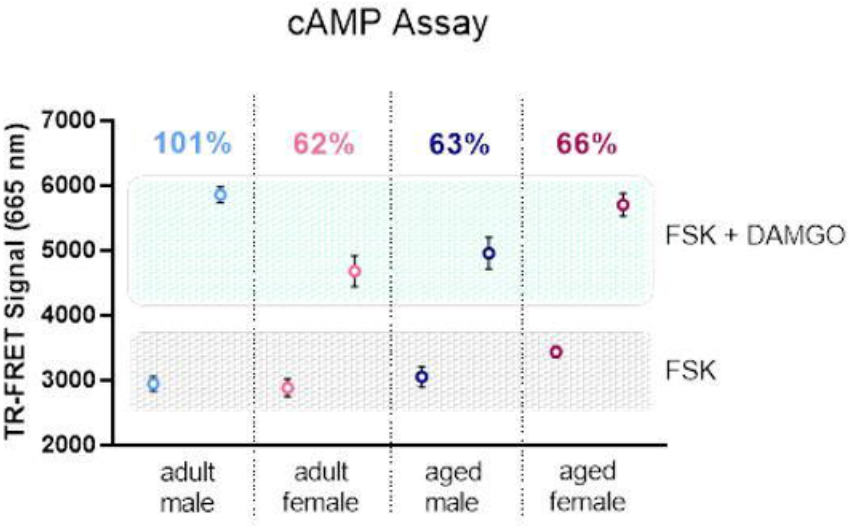

Phosphorylated MOR
ns
pMOR/tMOR
4
3
2
1
0
-0.1
34.0
0.02
male
female
male
female
adult
aged

Figure 4


cAMP Assay
TR-FRET Signal (665 nm)
7000
6000
5000
4000
3000
2000
101%
62%
63%
66%
FSK + DAMGO
FSK
adult male
adult female
aged male
aged female

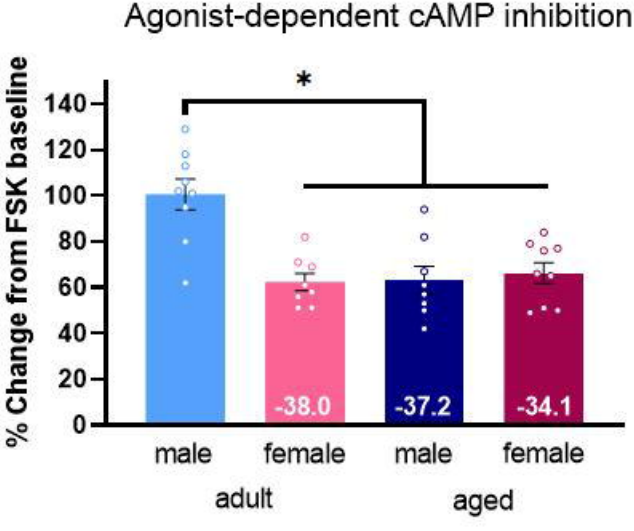
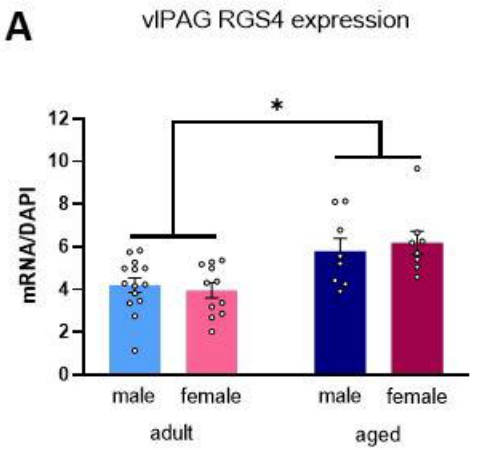
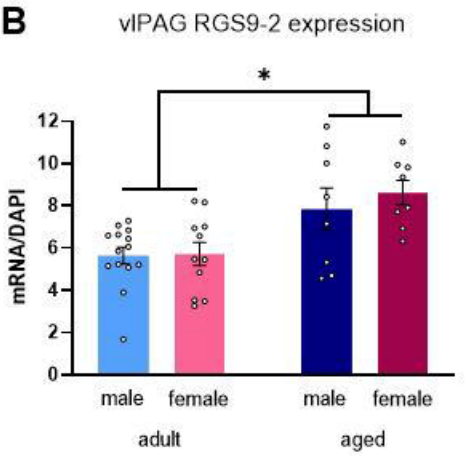
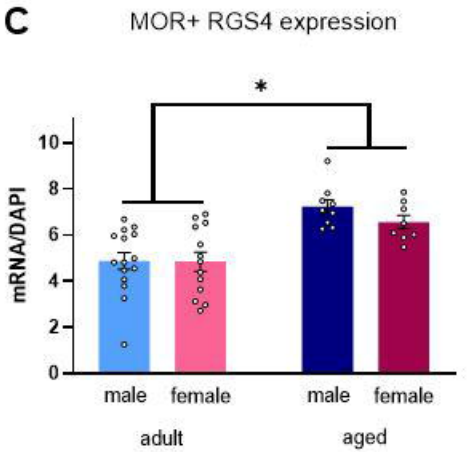
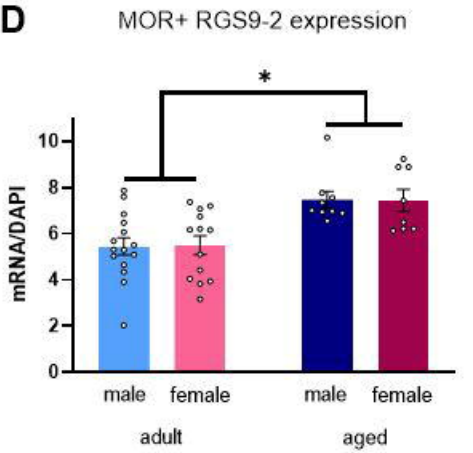

Agonist-dependent cAMP inhibition
% Change from FSK baseline
140
120
100
80
60
40
20
0
*
-38.0
-37.2
-34.1
male
female
male
female
adult
aged

Figure 5


A
vlPAG RGS4 expression
mRNA/DAPI
0
2
4
6
8
10
12
*
male
female
male
female
adult
aged


B
vlPAG RGS9-2 expression
mRNA/DAPI
0
2
4
6
8
10
12
*
male
female
male
female
adult
aged


C
MOR+ RGS4 expression
mRNA/DAPI
0
2
4
6
8
10
*
male
female
male
female
adult
aged


D
MOR+ RGS9-2 expression
mRNA/DAPI
0
2
4
6
8
10
*
male
female
male
female
adult
aged

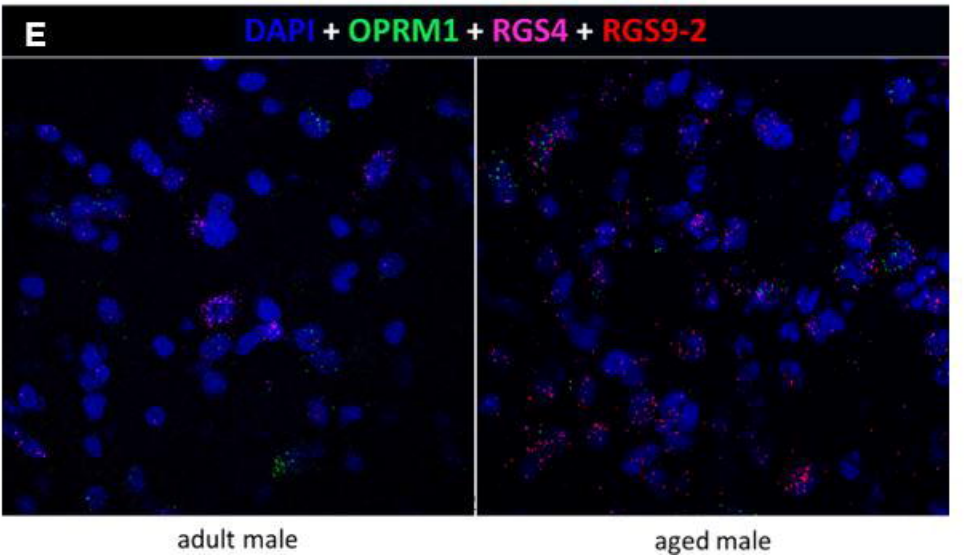
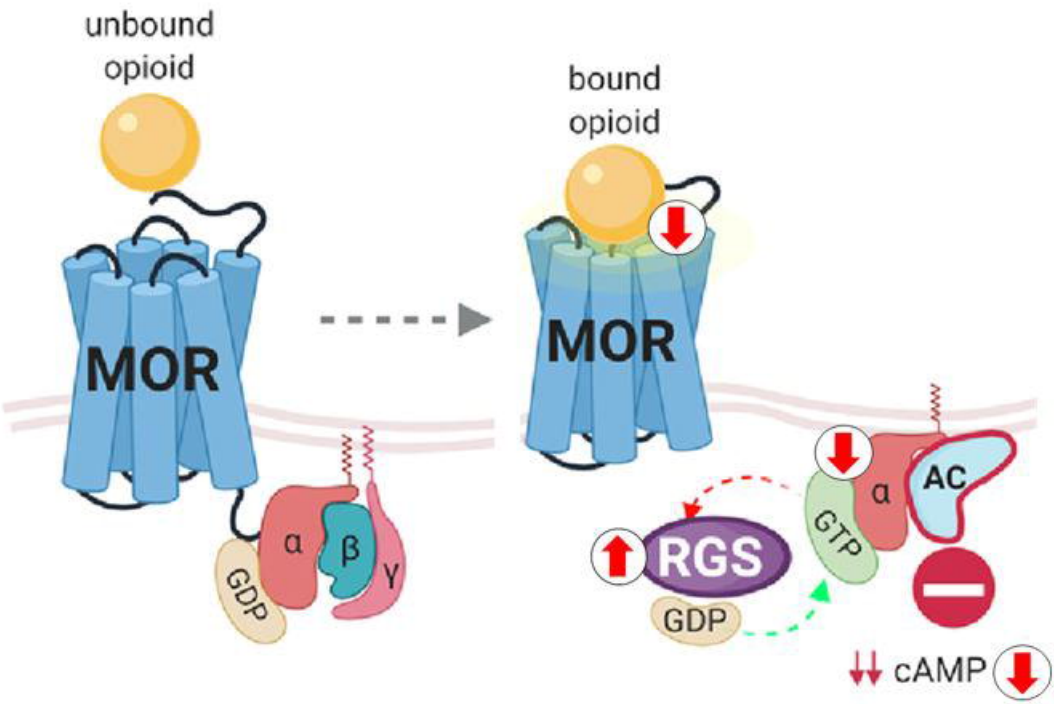

E
DAPI + OPRM1 + RGS4 + RGS9-2
adult male
aged male

Figure 6



	Morphine Potency	Binding Potential	G-Protein Activation	MOR Phosphor.	cAMP Inhibition	RGS mRNA
Adult ♂	=	=	=	=	=	=
Adult ♀	↓	↓	↓	=	↓	=
Aged ♂	↓	↓	↓	=	↓	↑
Aged ♀	↓	↓	↓	=	↓	↑